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<b>(54) Title:</b> PROCESSES AND MATERIALS FOR CARRYING OUT SPECIFIC BINDING ASSAYS		
<b>(57) Abstract</b>  <p>A process for carrying out a label-linked specific binding assay, e.g. an immunoassay, comprising the steps of: providing in an aqueous reaction zone a member of a specific binding pair to participate in the assay, linked to a label which either is a first coreagent which is reactive with a second coreagent in a reaction to produce a detectable assay result, or is a generator of said coreagent; providing a dispersed quantity of said second coreagent in said reaction, or of a generator of said coreagent, within a discretely bounded zone adjacent to said aqueous reaction zone and formed by a zone-forming carrier material, which also carries in immobilised form a member of said specific binding pair; providing in said aqueous reaction zone a consumer of one of said coreagents in said reaction, thereby to ensure the substantial absence of said coreagent in said aqueous reaction zone; wherein said discretely bounded zone comprises a barrier sufficient to prevent entry of the consumer reagent from said aqueous reaction zone into the bounded zone, thereby to separate the consumer reagent from the dispersed quantity of second coreagent or generator thereof in the bounded zone; and allowing the specific binding reactions to take place whereby the assay reaction results in the binding of a variable quantity, dependent on the quantity of labelled member of said specific binding pair directly or indirectly, to said immobilised specific binding material, so that said detectable assay result is produced by reaction of bound label or said coreagent generated by said bound label with said coreagent in said bounded zone.</p>		

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Processes and Materials for Carrying Out Specific Binding Assays

This invention relates to improvements in processes and materials for carrying out specific binding assays.

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Included among specific binding assays are the well-known immunoassays, as well as corresponding assay schemes in which, instead of an immunological pair, e.g., antigen or hapten and antibody, another kind of specific binding pair is involved, such as a hormone and its corresponding binding protein from serum, or biotin and avidin or streptavidin.

10

A variety of techniques is known for carrying out enzyme-linked and other forms of label-linked immunoassay, in which an enzyme or other label fixed to a member of a specific binding pair involved in the assay, has its activity determined as an index of the amount of labelled material present in the free or bound state. The prior art is rich in variety of patterns of reaction scheme and assay configuration, as well as in the variety of particular specific binding pairs involved in the assays. Illustrative examples of specifications describing such assays are: USP 3 654 090,

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USP 3 791 932, USP 4 376 110, GB 2 074 727,  
EP 0 014 530, and EP 0 042 755.

Also included in the prior art are devices for carrying  
5 out immunotests for antigens, in which reagent zones are  
arranged in the form of solid or fibrous layers containing  
various reagents needed for the tests (GB 2 111 676,  
(Liotta)). In the devices of GB 2 111 676, a layer of  
10 immobilised antigen intervenes between a layer where  
sample and enzyme-linked antibody are applied, and a  
colour-forming layer, so that in principle only the  
labelled antibodies bound to the sample can penetrate to  
the colour-forming layer.

15 Specification GB A 2 023 816 (Damon) describes immunotests  
in which an antibody for carrying out an immunotest of the  
separation type is occluded within semipermeable nylon  
microcapsules: the test is specific for a freely  
diffusible analyte (such as thyroxin or cortisol etc) and  
20 the free or bound portion after a binding reaction is  
estimated from the quantity of labelled material within or  
outside the capsules.

The prior art also contains many other clinical assay  
25 arrangements, e.g. glucose indicators as exemplified in  
GB 2 036 963 (Miles) and GB 2 026 160 (VEB  
Arzneimittelwerk Dresden), EP 0 058 334 (Miles) and  
EP 0 078 971 (Behringwerke), and the devices and tests of  
EP 0 110 173 (Lifescan), WO 84/02004 (Quidel) and  
30 WO 79/01081 (Battelle).

USP 4 233 402 (Syva) discloses so-called "enzyme  
channelling" immunoassays, which involve conjugates of two  
different enzyme labels, one with each of two  
35 complementary specific binding partners. The product of  
the first enzyme (e.g., hydrogen peroxide from glucose

oxidase) is a substrate for the second enzyme (e.g. horseradish peroxidase), and the molecular coupling between the two different enzyme molecules in the resulting immune complexes is mentioned as giving enhanced production of colour product. Further enzyme channelling assays are mentioned in "Monoclonal Antibodies and Developments in Immunoassay" (1981, Elsevier) pp 109-119, in an article entitled "Homogeneous Enzyme Immunoassay Techniques for Proteins", by E.F. Ullmann, in one example of which the conjugate of one enzyme and binding pair member is coupled to agarose beads, and catalase is supplied to consume hydrogen peroxide which escapes without reacting with bound enzyme, to minimise its reaction with unbound enzyme.

The present invention aims to provide a number of novel assay arrangements which can be widely applied to many different analytes, with a common reaction pattern which provides relative simplicity and convenience in use.

According to the invention there is provided a process for carrying out a label-linked specific binding assay, e.g. an immunoassay, comprising the steps of

- 25 - providing in an aqueous reaction zone a member of a specific binding pair to participate in the assay, linked to a label which either is a first coreagent which is reactive with a second coreagent in a reaction to produce a detectable assay result, or is a generator of said coreagent,
- 30
- providing a dispersed quantity of said second coreagent in said reaction, or of a generator of said coreagent, within a discretely bounded zone adjacent to said aqueous reaction zone and formed by a zone-forming carrier material, which also carries in
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immobilised form a member of said specific binding pair,

- 5       - providing in said aqueous reaction zone a consumer of one of said coreagents in said reaction, thereby to ensure the substantial absence of said coreagent in said aqueous reaction zone,
- 10       - wherein said discretely bounded zone comprises a barrier sufficient to prevent entry of the consumer reagent from said aqueous reaction zone into the bounded zone, thereby to separate the consumer reagent from the dispersed quantity of second coreagent or generator thereof in the bounded zone,  
15       and
- 20       - allowing the specific binding reactions to take place whereby the assay reaction results in the binding of a variable quantity, dependent on the quantity of analyte present in the assay system, if any, of said  
25       labelled member of said specific binding pair directly or indirectly to said immobilised specific binding material, so that said detectable assay result is produced by reaction of bound label or of coreagent generated by said bound label with said  
30       coreagent in said bounded zone.

Also provided by the invention is a test kit for carrying  
30       out a specific binding assay, e.g. an immunoassay, the test kit comprising

- 35       - a labelled member of a specific binding pair that participates in the binding reaction of interest in the assay, linked to a label material which either is a first coreagent which is reactive with a second

coreagent in a reaction to produce a detectable assay result, or is a generator of said first coreagent,

5 - a bounded disperse reagent preparation which  
comprises a zone-forming carrier material which  
contains within a discretely-bounded zone a dispersed  
quantity either of said second coreagent in said  
reaction, or of a generator of said second coreagent,  
10 said bounded disperse reagent preparation also  
carrying in immobilised form at the zone boundary  
formed by said zone-forming carrier material a member  
of said specific binding pair, and

15 - a consumer reagent capable of consuming one of said  
coreagents,

wherein the zone boundary formed by said zone-forming  
carrier material provides a barrier sufficient to  
prevent entry of the consumer reagent into said  
20 discretely-bounded zone, thereby to separate the  
consumer reagent from the dispersed quantity of  
second coreagent or generator thereof in said zone,

25 - wherein the bounded disperse reagent preparation and  
the labelled material and the consumer reagent are  
arranged so that they can be put into contact during  
performance of the assay, with the labelled material  
and the consumer reagent dispersed in an aqueous zone  
adjacent to the bounded disperse reagent preparation,  
30 whereby in use the consumer reagent ensures the  
substantial absence of one of said coreagents in said  
aqueous reaction zone, and the assay reaction results  
in the binding of a variable quantity of said  
labelled material directly or indirectly to said  
35 immobilised binding material, so that said detectable  
assay result is produced by reaction of said

coreagent in said bounded disperse reagent preparation with said label bound thereto or with coreagent generated by said label.

- 5 Several examples of the invention correspond to an assay system (and corresponding process and test kit) for carrying out a label-linked specific binding assay (e.g. an immunoassay),
- 10 wherein a label is used which either is a first coreagent which is reactive with a second coreagent in a reaction to produce a detectable result, or is a generator of said first coreagent,
- 15 said label being linked to a member of a specific binding pair relevant to the assay, to form a labelled specific binding material,
- 20 said assay system comprising a first zone containing a dispersed quantity of said second coreagent in said reaction, or a generator of said second coreagent, said first zone also carrying an immobilised member of said specific binding pair,
- 25 and a second (aqueous) zone comprising a consumer of one of the coreagents in said reaction, together with said labelled specific binding material,
- 30 whereby in use the assay reaction results in the binding of a variable quantity of said labelled member of said specific binding pair in said first zone, so that said detectable result is produced by reaction of the coreagent in the first zone with the labelling material or the coreagent generated by said labelling material.
- 35 In the assay system, the first coreagent can be for example a participant in a luminescent reaction, or an enzyme that catalyses directly or indirectly a luminescent or chromogenic or fluorogenic reaction, and the mentioned second coreagent is for example a further reactant in said reaction or an enzyme that catalyses said reaction.



A generator of said coreagent can be for example an enzyme that catalyses a reaction of which the product is said coreagent, or a system that generates said co-reagent non-enzymically.

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The first zone can be for example a discrete zone, for example that formed by a gel or capsule or bead, and the surface thereof, optionally together with the zone molecularly adjacent to said surface, i.e., that zone which contains any molecules directly or indirectly sorbed to said surface.

The immobilised member of said specific binding pair can for example be carried on said surface, and can be fixed there by per se known technique for immobilising specific binding materials.

The said second coreagent in said reaction (or generator thereof) which is dispersed in said first zone can in certain examples be occluded or entrapped there, e.g. by occlusion of free coreagent or generator within liposomes or membrane microcapsules or entrapment during polymerisation of a polymer such as acrylic polymer.

The boundary between the first and the second zone in examples described below is such as to prevent the consumer reagent (which can be for example an enzyme) from penetrating or dispersing or diffusing into said first zone, and in many cases also prevents the labelled specific binding material from penetrating further than the surface and/or the molecularly adjacent layer of the first zone.

The discretely-bounded zone can contain either an enzymic or non-enzymic generator of the second coreagent, or a supply of the second coreagent.

According to the present invention, there is also provided a process for carrying out a label-linked specific binding assay, e.g. an immunoassay, comprising the steps of (1) providing an assay system having a first zone in which there is present an amount of a reactant effective to react with the label to give directly or indirectly a detectable result, and an adjacent second zone in which the reactant is effectively absent, (2) providing the label in a form in which it is linked to a member of the specific binding pair appropriate to the specificity of the assay in the second zone in which the reactant is effectively absent, (3) providing an immobilised specific binding partner for the material which is linked to the label in the first zone in which the reactant is effectively present, (4) allowing the binding partners to undergo binding reactions in the presence of an analyte sample, whereby the label-linked material becomes bound to its binding partner in the first zone in an amount which depends on the quantity of analyte present in the assay system, and (5) detecting the result of reaction of the label with the reactant in the first zone.

A test kit according to a corresponding embodiment of the invention, for carrying out a specific binding assay, comprises a first reagent preparation, and a second reagent preparation, which in admixture provide an assay system having a first zone and an adjacent second zone, wherein said first reagent preparation comprises an immobilised specific binding material with a specificity appropriate to the desired assay, and a reactant supply system to supply or generate a reactant in said first zone wherein said immobilised specific binding material is located, which reactant is capable of reacting with an assay label to produce a detectable result, and wherein said second reagent preparation comprises a specific binding material with a specificity appropriate to the

desired assay, linked to a label which is capable of reacting with said reactant, the test kit also comprising a consumer reagent to be located in said second zone to consume any reactant which in use may arrive there by transfer from said first zone, and optionally also a coreactant to react with said label with said reactant to produce said detectable result.

In particular examples of the test kit, said first reagent preparation comprises a preparation of beads or capsules to provide the first zone, carrying immobilised specific binding material and containing a hydrogen-peroxide-generating system as reactant-generating system, and said second reagent preparation comprises specific binding material linked to a peroxidase enzyme or a peroxide-reactive chemiluminescent label material, the test kit also comprising catalase (in use to consume any hydrogen peroxide in the second zone outside the beads or capsules), and optionally also a chromogenic or fluorogenic or luminescent material to give a detectable result upon reaction with the label and the reactant.

Usually, the label is brought into contact with the reactant in the first zone by arranging that there is a determinate quantity of specific binding material located in immobilised form in the first zone, to bind a variable quantity of the label linked to one of the specific binding partners in the assay reaction, according to the quantity of analyte present in the test system.

According to a convenient arrangement within the scope of the invention, the reactant is diffusible, is supplied or provided in the first zone, and its effective absence from the second zone is assured by a consumer reagent in the second zone that effectively consumes the reactant diffused from the first zone, i.e. to prevent it from

undergoing appreciable or substantial reaction with the label in the second zone.

The label and the reactant are chosen so that they can  
5 react together in the first zone to produce a detectable  
result. In many examples, a suitable label is for  
example an enzyme, and a detectable product results from  
reaction of the enzyme and the reactant, e.g. a coloured  
or fluorescent product, or a result that is detectable in  
10 some other way, e.g. as a luminescent reaction. In many  
examples of this system, a co-reactant is present, which  
reacts with the first reactant and enzyme to produce the  
product. (The first reactant can be for example a  
peroxide such as hydrogen peroxide, the co-reactant can be  
15 a chromogen or fluorogen or a reactant in a luminescent  
reaction, and the enzyme can be a peroxidase.) Any  
co-reactant in the system can (but need not) be localised  
to the first zone: it can for example be soluble and  
present in solution in aqueous liquid forming the second  
20 zone.

An alternative suitable enzyme, to be occluded within gel  
or microcapsules, is yeast methanol oxidase, supplied with  
substrate quantities of methanol and oxygen, thereby to  
25 supply hydrogen peroxide as a reactant for the assay.

In alternative embodiments, the label need not be an  
enzyme: it can for example be a luminescent substance.  
A suitable example of such a substance is an N-methylated  
30 product of a 9-acridine-carboxyl group, which reacts with  
 $H_2O_2$  as the reactant, to give a light output. Where  
appropriate, references to "enzyme" herein include the  
case of another type of reactive label.

35 These arrangements can for example be provided by means of  
a biphasic or two-compartment system in which diffusible

reactant is supplied or provided, e.g. by a reactant-generating system, in the first phase or compartment and diffuses into the second phase or compartment where it is consumed by the consumer reagent.

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This arrangement offers the advantage of example of enabling the production of immunoassays in which the result is indicated by a colour reaction, with unusually definite endpoints at high or low analyte levels, and very few liquid addition operations. Often the minimum rate of colour formation attainable in a chromogenic immunoassay is an appreciable fraction of the maximum rate of colour formation, and many liquid addition operations are needed.

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15 In a further alternative embodiment, the first or bounded zone of the reaction system can contain a chromogenic, fluorogenic or luminescent coreagent or plural number of coreagents, e.g., horseradish peroxidase with one of its substrates mentioned herein, carried in for example a  
20 polymer gel which is sufficiently crosslinked to exclude entry of catalase, and carrying specific binding activity fixed to the gel surface, and the label can be a generator of a remaining coreagent, e.g. glucose oxidase to generate hydrogen peroxide. In this arrangement the unbound  
25 labelled material generates hydrogen peroxide coreagent in the aqueous liquid adjacent to the gel, but the consumer reagent (catalase) consumes it. Upon binding of the labelled material to the gel surface the generated coreagent can produce a detectable result by reaction with  
30 the materials contained within the gel.

The provision of a consumer reagent or otherwise of a reactant-free zone enables the minimum rate of colour formation to be brought down much lower than usual and  
35 ideally to zero.

- A further advantage of this assay arrangement lies in the facility to provide all reagents together in one reaction volume. There is no need to separate free labelled material from bound labelled material, nor to separate the reaction liquid for the specific binding step(s) from the reaction liquid in which a chromogenic or fluorogenic enzyme reaction takes place in order to enable detection of the assay result.
- Using the techniques described herein, it is possible and advantageous to add in a single reaction liquid, or reaction system all the components of the assay including the chromogen (or fluorogen or other co-reactant such as luminescent material).
- The first zone of the assay system of the invention can effectively comprise the first phase or compartment of the biphasic or two-compartment system mentioned above, together with so much of the interphase or interfacial region as contains appreciable supplies of the reactant, and the second zone effectively comprises the rest of the second phase or compartment.
- Under these conditions, the enzyme can be brought into contact with the reactant by a binding event which fixes it in the interphase or interfacial zone. For example, the interfacial or interphase zone can include a gel surface or a membrane having fixed thereto specific binding sites to which the enzyme can become bound, e.g. in the form of a conjugate with a member of the specific binding pair relevant to the assay, to an extent dependent on the quantity (if any) of analyte (which can be one member of the specific binding pair) present in the system. The specific binding sites can be provided by (for example) per se conventional techniques for fabrication of immunoabsorbents.

The way in which the enzyme becomes bound can correspond to any of the ways in which enzymes have heretofore been bound to immunoabsorbents during the course of immunoassay. For example, in several arrangements an

5 enzyme linked to an antigen or hapten can become bound to an antibody immunosorbent in inverse relation to the quantity of analyte antibody (or antigen or hapten) which is available for previous binding or completion with respectively the enzyme conjugate or the immunoabsorbent.

10 This is the pattern that occurs in for example a competitive immunoassay. Alternatively the enzyme linked to an antibody or antigen can become bound to immunosorbent in direct proportion to the quantity of analyte that can be bound both by the immunosorbent and by

15 the conjugate although these latter do not otherwise appreciably bind with each other. This is the pattern that occurs in for example a "sandwich" immunoassay or an antiglobulin test or an immunoglobulin capture assay.

20 For example, a competition assay can be arranged by attachment of antibody to the interphase or interfacial zone, e.g. anti-beta(2)microglobulin or anti-C-reactive protein, and the enzyme to be brought into effective contact with the reactant can be provided in a form in

25 which it is conjugated, by any suitable known conjugation technique, to the corresponding antigen, namely beta(2)microglobulin or C-reactive protein. The analyte in such a case can be the corresponding antigen itself.

30 By way of further example, a sandwich assay can be arranged again by attachment of antibody to the interphase or interfacial zone, e.g. anti-ferritin or anti-(hepatitis B surface antigen) or anti-(immunoglobulin E). A further antibody also with specificity for the same antigen can

35 then be used in the form of its conjugate with the enzyme to be brought into effective contact with the reactant.

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It is within the scope of the invention to link the label to the specific binding material or bindable material in an indirect manner, e.g. as a label occluded in a preparation of microcapsules or liposomes (such as those  
5 described in EP 0 014 530) and/or a label linked to a further binding material (such as an antiglobulin) with affinity for the material which it is intended to label.

It will be apparent to the reader that any specificity can  
10 be imparted to the assay materials provided by this invention upon appropriate choice of specific binding components.

One convenient method of providing an interphase or  
15 interfacial zone including an immunosorbent capacity is to provide beads or capsules containing a reactant-generating system together with an immunosorbent surface. For example, polyacrylamide beads containing a reactant-generating system can be provided with  
20 immunosorbent surfaces.

In one very suitable example of a reactant-generating system, an enzyme to generate the reactant is immobilised or occluded in the first zone, e.g. dispersed within a  
25 bead or capsule, e.g. a crosslinked polyacrylamide bead, but unable itself to escape into the second zone, which can be liquid surrounding the bead or capsule. A suitable example preparation of beads or capsules for this purpose comprises beads of 20-500 micron diameter,  
30 sufficient to allow free diffusion of low molecular weight reactants, e.g. made by suspension or emulsion polymerisation of acrylamide with crosslinker.

In alternative examples, the reactant-generating system  
35 can be contained within liposomes bearing specific binding material on their surface, e.g. those mentioned in EP



specification No. 0 014 530 and references cited therein, and examples given herein below.

A suitable enzyme to generate the reactant is for example  
5 glucose oxidase (provided with glucose and oxygen), which enzyme can be for example occluded within a polyacrylamide bead which has a degree of crosslinking sufficient to prevent any appreciable diffusion of the glucose oxidase out of the bead. The occluded glucose oxidase enzyme can  
10 be provided with its substrates glucose and dissolved oxygen by for example diffusion from outside the first zone, e.g. by diffusion from liquid surrounding the bead or capsule. This system generates hydrogen peroxide ( $H_2O_2$ ) within the bead, and the  $H_2O_2$  diffuses out freely  
15 from the bead into the surrounding liquid, where it can be used as the reactant. This example of a reactant for the rest of the assay system ( $H_2O_2$ ) can alternatively be produced by any other convenient generating source in the first zone, and diffuses through the interphase or  
20 interfacial zone into the second zone, e.g. surrounding liquid, where the consumer reagent can for example be a suitably chosen concentration of catalase enzyme.

An alternative analogous reactant is for example  
25 glucose-6-phosphate, which can be released for example from a gel preparation (as the first zone) which occludes a sufficient supply of glucose-6-phosphate, e.g. in solid slowly-dissolving form. For this purpose the consumer reagent present outside the first zone can be.  
30 glucose-6-phosphatase, and a corresponding enzyme label is glucose-6-phosphate dehydrogenase. In this case the coenzyme NADP is supplied, and the extent of its reduction can be detected in the usual (e.g. spectrophotometric or fluorometric) manner, and is directly related to the  
35 amount of the enzyme which has become bound to the gel surface.

The glucose-6-phosphate can be provided for example in the form of a finely-divided solid carried within a hydratable polymer gel, e.g., an acrylic polymer gel which is sufficiently crosslinked to exclude the consumer reagent, e.g. such a gel which has been produced by polymerisation of an acrylic comonomer mixture carrying the finely-divided solid particles in suspension, in non-aqueous solvent.

Conversion of such beads to specific binding material of desired specificity can be achieved in any suitable way, but in particular by incorporating biotin, e.g. in the manner described below, or a hapten, and then absorbing the biotin or hapten with avidin or anti-hapten conjugated to an antibody, etc., of wanted specificity.

Alternative reactant-generating systems not involving enzymes can also be chosen. For example, where hydrogen peroxide is the reactant which it is desired to generate, the reactant-generating system can be a chemical hydrogen-peroxide generating system, e.g. a photochemical system, in which the first zone is illuminated with light which is actinic for material which can reduce oxygen under photo activation in the presence of an electron-donor, e.g. flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), anthraquinone, methyl viologen or 1,1'-ethylene-2,2'-bipyridylum in the presence of an aliphatic tertiary diamine, such as a substituted ethylene diamine, e.g. EDTA. These photochemical hydrogen-peroxide-generating systems include systems known per se. It can be useful to add superoxide dismutase enzyme in these systems to lower the concentration of photochemically-produced superoxide which might affect some of the other reagents in the system.

It is apparent tht this invention is susceptible of a wide range of variations and modifications within its scope. Preferred embodiments of the invention are described by way of example only, together with details for the  
5 preparation or procurement of the principal assay materials, as follows:

Example 1

10 1. Preparation of principal assay materials:

A polymerisable biotin derivative, the 2-hydroxyethyl methacrylate ester of biotin, can be prepared by the following process:-

15

(a) Add 10ml of thionyl chloride to 1g of d-biotin (from Sigma Chemical Co. (Trade Mark)), keeping the temperature in the range 0-5°C. Leave for 1 hour with occasional shaking. Filter off the precipitate formed, and separate  
20 any unprecipitated product from the filtrate by adding 20ml of a 1:1 toluene-petroleum ether (100-120°) mixture. Wash the combined precipitates with a further 20ml of the solvent mixture.

25 (b) Use the biotinyl chloride product for the next stage immediately. Store any unused product over  $P_2O_5$  after vacuum oven drying at 25°C.

Dissolve 0.5g of the biotinyl chloride in a mixture of  
30 42.5g of 2-hydroxyethylmethacrylate (HEMA) and 0.5g pyridine. Adjust the pH to approximately 7 by the addition of more pyridine. Stir at 20°C for two hours. Add the mixture to 400ml toluene, and then add 400 ml water. Shake, and then remove the organic layer. Add a  
35 further 400 ml water and repeat the extraction. Dry the organic layer over anhydrous magnesium sulphate. Remove

toluene on a rotary evaporator, keeping the temperature less than 60°C. Store the product at 0-5°C. It contains the 2-hydroxyethylmethacrylate ester of biotin (biotin-HEMA ester) monomer in admixture with a  
5 substantial amount of HEMA.

(c) The following description shows use of the biotin-HEMA monomer prepared as described in parts (a) and (b) above to prepare copolymers with acrylamide and  
10 methylenebisacrylamide in bead form containing (occluded) active glucose oxidase enzyme, and having covalently-attached biotin groups at the surface, derived from the polymerisable biotin derivative comonomer. The polymer is sufficiently cross-linked to prevent  
15 avidin-enzyme (HRPO) conjugates from entering by penetrative diffusion, and to prevent the occluded enzyme from diffusing out, when the beads are suspended in aqueous liquid.

(d) Dissolve 7g acrylamide and 0.48g  
20 methylenebisacrylamide (MBA) in 32ml of sodium acetate buffer (SAB, pH 5.6) and then add 0.6g of the product from the biotin-HEMA monomer preparation (parts (a) and (b) above). (SAB contains 2.84ml acetic acid (glacial) +  
25 99ml water, and is adjusted in pH to 5.6 with sodium hydroxide solution, and diluted to 1 litre with water).

Deoxygenate by bubbling nitrogen through the mixture for fifteen minutes. Then add 3ml of an ammonium persulphate  
30 solution (0.4g/ml) and 8ml of a glucose oxidase solution (2mg/ml, in SAB). Pour the mixture into a 500ml glass bowl containing 350ml n-hexane and 0.9g SPAN 80 detergent (Trade Mark).

35 Stir the mixture with an overhead stirrer fitted with a "U" shaped glass stirrer contoured to the bottom and sides

of the bowl. Adjust the speed to approximately 220 rpm. Stir for one minute after the addition of the monomer mixture. Then increase the speed to about 250 rpm. Add 2.2ml tetramethylethylenediamine (TEMED) and continue  
5 stirring for three minutes. Turn the speed back down to 220 rpm and continue for one hour. (The bowl should be covered during stirring to reduce evaporation of hexane).

Decant off most of the hexane, filter off the beads, and  
10 wash with a large volume of SAB. Remove any large lumps of polymer, and store in SAB with a small amount of thimerosal (antimicrobial preservative) at 0-5°C.

Examples of beads prepared according to the above  
15 procedure have been found to have a particle size range of about 30-500 microns and to show swelling (in SAB) of 5.1g/g and glucose oxidase activity equivalent to about  $1.3 \times 10^{-4}$ g (enzyme)/g (dry polymer).

20 (e) An alternative polymer bead preparation, similar to that described in part (d) but containing a lower proportion of biotin groups, can be made as follows. The procedure is the same as described in part (a) above, except for the following modifications. The monomer  
25 mixture is made with 1ml of a solution of the biotin-HEMA monomer product in water ( $6.6 \times 10^{-3}$ g/ml + 7g acrylamide + 0.48g MBA + 32ml SAB. Examples of beads prepared according to this modified procedure have shown a swelling in SAB of 5.3g/g and a glucose oxidase activity equivalent  
30 to about  $1.1 \times 10^{-4}$ g (enzyme)/g (dry polymer).

The following details refer to an assay for biotin, using among other materials avidin-peroxidase conjugate. It will, however, be clear to the skilled reader that the  
35 polymer beads prepared as described above can be converted to form an immunoadsorbent of desired specificity by first

- 20 -

adsorbing them with a conjugate of avidin or streptavidin linked in known manner (e.g. by use of a known glutaraldehyde coupling technique) to an appropriate antigen or antibody or specific binding analogue or  
5 specific binding competitor of an antigen or antibody of appropriate specificity.

Then the desired form of immunoassay or other specific binding assay can be arranged by using the peroxidase  
10 conjugate of an appropriate specific binding agent in place of the avidin-peroxidase conjugate used as described below. The format of the immunoassay can be chosen from among any of those described hereinabove, or any other suitable assay format.

15

## 2. Performance of assay:

Materials for the performance of an example specific binding assay include:

20

- (a) polymer beads with covalently attached biotin and occluded glucose oxidase, as prepared in parts 1(a)-(d) above;
- (b) a conjugate of horseradish peroxidase (HRPO) with  
25 avidin D, either as obtained commercially (Sera-Lab or Vector Laboratories (Trade Marks), protein concentration 5-10 mg/ml, ratio HRPO:avidin 1.5:1 to 2:1) or as prepared for example by the known glutaraldehyde coupling technique from HRPO and avidin;
- (c) catalase (bovine liver catalase, thymol-free, from  
30 Sigma Chemical Co. (Trade Mark));
- (d) D-glucose;
- (e) 2,2'-azinodi-(3-ethylbenzthiazolinosulphonic acid) diammonium salt (from Sigma Chemical Co. (Trade Mark))
- 35 (ABTS or ADBS);

- (f) a liquid sample containing an unknown quantity of biotin, diluted if need be to supply of the order of 0.001-0.1 $\mu$ g biotin per ml of final reaction liquid. (The precise useful assay range is calibrated in the normal way against standards, for each particular manner of preparation of the assay reagents).

Component (b), the conjugate, is used in diluted admixture with an inert protein, (ratio 200-400 $\mu$ g conjugate to 100mg protein), in this case bovine serum albumin, in order to stabilise it in the usual stock solutions and containers.

An assay of biotin using these materials can be performed either in two stages or in one stage. To perform the assay in two stages, a first stage mixture can be made, comprising the following constituents (per ml of reaction liquid): (i) 50mg of bead component (a); (ii) a standard aliquot of conjugate (b) in a quantity in the range about 7.5-15 $\mu$ g which is the same for all samples, calibration standards and controls in a given assay run; (iii) 3.75mg bovine serum albumin, (diluent/stabiliser, inert protein used in admixture with the stock solutions and preparations of conjugate (b)). The liquid in which stock solutions are made up for suspending and dissolving these components is phosphate-buffered saline (PBS) (containing (per litre) 8.5g NaCl, 1.07g disodium phosphate and 0.39g monosodium phosphate in purified water); and (iv) sample liquid adjusted if possible (e.g. by serial dilution) to contain about 0.003-0.3 $\mu$ g biotin.

The first stage mixture can be shaken for about 40 minutes to keep the polymer beads in suspension. Then the beads are separated, e.g. by centrifugation, and added (sample by sample) to respective aliquots of further reagents to make a second stage mixture containing (per 2.5ml); 50mg of treated beads from first stage mixture, 0.46mg

catalase, 2mg ABTS, 10mg glucose, all in PBS solution. After 30 minutes' incubation an aliquot of supernatant liquid is taken for absorbance measurement at 630nm against a comparison blank of PBS solution containing only  
5 the ABTS, catalase, and glucose as used in the second stage reaction mixture.

The assay routine has been found to permit satisfactory assay of biotin at the levels indicated.

10

In alternative assays, all the reagents are mixed together in a single reaction liquid (the weights of reagents given above for the second stage can be scaled down to leave the same final concentrations). This enables a particularly  
15 convenient form of one-stage assay which can be presented by means of appropriate combinations of stock reagents so as to minimise the number of manipulations required on the part of the user.

20 Such an assay can be carried out for example as follows. In the following example procedure the binding reaction partners are allowed to react together before the addition of substrate and consumer enzyme (catalase), but experiments have shown that this delayed addition is  
25 unnecessary: the substrate and the enzyme can be added at the start.

Stock solutions of avidin-HRP conjugate (obtainable from Vector Laboratories (Trade Mark)) are prepared as follows:

30

- (a) 7.5  $\mu$ l avidin-HRP conjugate + 18.8 mg bovine serum albumin (BSA);
- (b) 7.5  $\mu$ l avidin-HRP conjugate + 18.8 mg BSA, + 7.5  $\mu$ l biotin solution;
- 35 (c) 7.5 $\mu$ l av-HRP conjugate + 18.8 mg BSA + 75  $\mu$ l biotin solution;



- (d) 7.5  $\mu$ l av-HRP conjugate + 18.8 mg BSA, + 150  $\mu$ l biotin solution;
- (e) 7.5  $\mu$ l av-HRP conjugate + 18.8 mg BSA, + 225  $\mu$ l biotin solution;
- 5 (f) 7.5  $\mu$ l av-HRP conjugate + 18.8 mg BSA, + 375  $\mu$ l biotin solution;
- (g) 7.5  $\mu$ l av-HRP conjugate + 18.8 mg BSA, + 563  $\mu$ l biotin solution;
- (h) 7.5  $\mu$ l av-HRP conjugate + 18.8 mg BSA, + 750  $\mu$ l biotin solution.
- 10

Biotin solutions were  $4.2 \times 10^{-4}$  mg/ml in phosphate buffered saline (PBS). All the above stock solutions are diluted to 5 ml with PBS before use.

15

The biotin-substituted beads, (high biotin level) are filtered and weighed out into 24 x 50 mg lots. These are divided into eight lots of three and 1 ml of solution (a) is added to each bead sample of one group of three, 1 ml of (b) to each sample of another group of three, etc. (to give triplicate readings at each point). They are then put on a wrist shaker for 40 minutes.

20

To each bead sample 1 ml catalase solution (0.46 mg/ml, in PBS) is added, then 1 ml ABTS dye solution (2 mg/ml, in PBS) and then 0.5 ml glucose solution (20 mg/ml, in PBS). They are put on a wrist shaker.

25

Thirty minutes after the glucose addition, 200  $\mu$ l of supernatant is removed (after having allowed the beads to settle). The optical densities are measured at 630 nm against a blank composed of 1 ml PBS + 1 ml catalase solution + 1 ml dye solution + 0.5 ml glucose solution.

30

Within the range of 0.6 - 35 nanogram/tube biotin the colour development of the assay tubes was found to vary

35

between substantially its maximum and minimum values, inversely with the biotin concentration.

### Example 2

5

In this example, gentamicin is assayed by a procedure in many ways analogous to the procedure given above for Example 1.

- 10 The components of an illustrative microbead assay system for the assay of gentamicin are in outline as follows (further description is given below):-

	<u>Component</u>	<u>Description</u>	<u>Quantity (per tube)</u> <u>= 250 <math>\mu</math>l total)</u>
15	Micro-beads	Polyacrylamide (cross-linked) with entrapped glucose oxidase and bearing on their surface sheep-anti-gentamicin antibodies	12.5 mg (wet wt.)
20			
	HRP-gentamicin	Prepared by periodate oxidation of HRP and subsequent reaction with gentamicin	665 ng HRP eqvt.
25			
	Catalase	Bovine liver, ex Sigma, (Trade Mark), thymol free, 10-25,000 units/mg	100 $\mu$ g
30			
	Buffer	Phosphate/citrate, pH 6.1 + Triton x 100 (1% v/v)	Used throughout
35			

Substrate            Glucose (0.1 M)            4 mg  
                      O-phenylene diamine        160 µg  
5                       (product is measured  
                      as O.D. at 405 nm)

Samples             In range from 0.2-15    50 µl  
                      mg/litre

10

The following section describes the preparation and characterisation of H<sub>2</sub>O<sub>2</sub>-generating beads for the gentamicin assay and the preparation of HRP-conjugates. The example bead types prepared in this way all contain  
15 active glucose oxidase: but they can differ in the functional groups arrayed on their surfaces. Beads produced for use with the avidin-HRP conjugate (substantially as in the preceding example) are given surface biotin groups, whilst beads intended for linking  
20 to amino groups on antibodies or antigens are provided with carboxyl groups (through the incorporation of acrylic acid as a comonomer).

The coupling of antibodies to carboxyl-containing  
25 beads can be accomplished in essentially known manner by means of the N-hydroxy succinimide/EDC (carbodiimide) reaction.

#### Preparation of acrylamide:sodium acrylate beads

30

Beads to be used for coupling via the amino groups of the antibody are prepared using sodium acrylate comonomer to give surface carboxyl groups, (in the following modification of the procedure described above for  
35 biotin-substituted beads in connexion with Example 1).

The materials used include: Sodium carbonate (anhydrous); PBS buffer (pH 7.2); Acrylic acid (glacial); Acrylamide and N,N<sup>1</sup>-methylenebisacrylamide (MBA), electrophoresis grade; Glucose oxidase, type VII ex Sigma; Ammonium persulphate; Hexane (fraction from petroleum); SPAN 80 (Trade Mark) surfactant; Tetramethylenethlenediamine (TEMED), electrophoresis grade; Thimerosal.

The suspension medium is prepared by dissolving SPAN 80 (0.4g) in hexane (350 ml) in a round bottomed glass reaction flask. The monomer solution is prepared by dissolving sodium carbonate (0.88g) in PBS buffer (33.3g). To this glacial acrylic acid is slowly added (1.19g) and the solution was stirred until effervescence ceases. Acrylamide (4.70g) and methylenebisacrylamide (0.93g) are added and allowed to dissolve. The solution is deoxygenated by bubbling with nitrogen for ten minutes. 1 ml glucose oxidase solution (15 mg/ml in distilled water) is dispersed in the monomer solution using the nitrogen stream to obtain good mixing. 1.5 ml ammonium persulphate solution (0.4 g/ml in distilled water) is added the nitrogen stream to thoroughly mix this in, over a 30 second period. The monomer mixture is poured into the warmed suspension medium (27-29°C) whilst stirring with a U-shped glass stirrer at approximately 230 rpm. After 30 seconds 5.0 ml TEMED is added to the hexane and the flask is covered to minimise solvent loss through evaporation. The polymerisation is allowed to proceed for 30 minutes, then the stirrer speed turned down to approx. 120 rpm for a further 25 minutes. The stirrer is then stopped to allow the beads to settle. Most of the hexane is decanted off, and the beads are filtered and washed with PBS until free of both hexane and SPAN 80 surfactant. The beads are stored as a slurry in PBS (4°C) containing approx. 0.01% w/v thimerosal as preservative.

The resulting beads, as made in one example test run of this procedure, were found to contain glucose oxidase activity at the level of 0.140  $\mu$ g enzyme/mg dry beads.

- 5 Anti-gentamicin antibodies are coupled to the micro-beads for example as follows.

Materials used include: Micro-beads bearing surface  
carboxyl groups (prepared as above), 5g (wet weight);  
10 N-hydroxy succinimide (NHS), 70 mg;  
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) 100  
mg; IgG fraction of sheep anti-gentamicin serum, 2mg;  
0.1 M bicarbonate buffer (pH 9.8) containing 0.3% (v/v)  
ethanolamine; 0.1 M phosphate buffered saline, pH 7.4  
15 (per litre: 85g NaCl, 1.07g sodium hydrogen phosphate and  
0.39g disodium phosphate adjusted to pH); "PC"  
Phosphate/citrate buffer pH 6.1 (containing 7.75 g/l  
citric acid and 22.48 g/l disodium phosphate per litre);  
and  
20 "PCT" Phosphate/citrate buffer pH 6.1; containing + 1%  
v/v Triton X-100 (Trade Mark) surfactant.

The micro-beads are washed in distilled water to remove  
preservatives, etc. The washed microbeads (5g, wet wt.)  
25 are resuspended in 6 ml distilled water. NHS (70 mg) in  
5 ml distilled water is added to the suspension so formed.  
EDC (100 mg) in 5 ml distilled water is then added,  
bringing the total volume to about 20 ml. The reaction  
mixture is roll-mixed for 1 hour at room temperature  
30 (activation). After 1 hour the micro-beads are washed  
with 3 x 15 ml distilled water. The washed, activated  
micro-beads are resuspended in 10 ml distilled water  
containing 3 mg anti-gentamicin IgG fraction. This  
activated micro-bead/IgG reaction mixture is roll-mixed  
35 overnight at 4°C to couple the antibody to the activated  
microbeads. The antibody-coupled micro-beads are then

- 28 -

washed with 2 x 15 ml distilled water. Ethanolamine (0.3% v/v) in 15 ml 0.1 M bicarbonate buffer (pH 9.8) is then added to the antibody coupled micro-beads to neutralise (block) residual protein-coupling sites on the  
5 beads. The reaction mixture is roll-mixed for 1 hour at room temperature. After 1 hour the micro-beads are washed in 1 x 15 ml distilled water, 2 x 15 ml PBS pH 7.4, 1 x 15 ml phosphate/citrate buffer, pH 6.1 + 1% Triton, 1 x 15 ml phosphate/citrate buffer, pH 6.1 and, finally they  
10 are suspended in 15 ml of phosphate/citrate pH 6.1. The beads are then stored at 4°C in phosphate/citrate buffer, pH 6.1.

Gentamicin-HRP Conjugate is prepared by a technique  
15 adapted from the description by Boersma and Streefkerk (J. Immunol. Methods., 1979, 30, 245-255):-

Materials used include:- Horse radish peroxidase (HRP, Sigma type VI) (10 mg); Gentamicin sulphate (2.8 mg);  
20 Sodium m-periodate, 0.1 M aqueous solution (500 µl); Sodium borohydride (400 µg); Visking tubing (narrow bore); 0.1 M acetate buffer, pH 4.0; 1 M carbonate buffer, pH 9.5; 0.01 M phosphate buffered saline pH 7.4 (PBS).

25 HRP (10 mg) is dissolved in distilled water (2 ml). Sodium m-periodate (0.1 M aqueous 500 µl) is then added. This reaction mixture is rolled for 25 minutes at room temperature to oxidise sugar groups on the enzyme. After  
30 25 minutes rolling, the mixture is dialysed overnight at 4°C against 0.1 M acetate buffer (pH 4.0). The dialysed mixture is then transferred to a reaction vial with gentamicin sulphate (2.8 mg) in 1M carbonate buffer (pH 9.5, 1 ml). This reaction mixture is rolled for 2 hours  
35 at room temperature (to couple gentamicin to HRP by Schiff base formation). After 2 hours rolling, sodium

borohydride (400 µg) in distilled water (100 µl) is added. The reaction mixture is rolled for 2 hours at 4°C.

After 2 hours rolling the mixture is transferred to narrow bore Visking (Trade Mark) tubing and dialysed overnight at 4°C against 0.01 M PBS, pH 7.4. Finally, the product (conjugate) is stored at 4°C in a stoppered brown glass vial (5 ml capacity).

Using the materials prepared above, a competitive assay for gentamicin can be carried out as follows. In this system antibodies are bound to the bead surface, and the labelled material used is gentamicin labelled with horse radish peroxidase (as described above). Free gentamicin from the sample is able to compete for the antibodies bound to the bead surface.

Materials used for assay include:-

Antibody-linked micro-beads (12.5 mg/tube); Gentamicin-HRP conjugate; Gentamicin sulphate solution (6 µg/ml in PCT Buffer); Catalase solution (in PCT), 2,000 µg/ml (ex Sigma, bovine liver, thymol free, 10-25,000 units per mg); D-glucose, 100 mg, and O-phenylenediamine, 8 mg, in 10 ml PC Buffer, pH 6.1; Phosphate citrate buffer, pH 6.1 (PC buffer); 0.01M phosphate buffered saline, pH 7.4 (PBS); Phosphate citrate buffer, pH 6.1 + Triton x 100 (PCT).

A set of tubes (2 ml Sarstedt) is arranged, each containing 50 µl of antibody-linked micro-beads (= 100 µg per tube). Gentamicin standards (50 µl each) are then added to give replicate tubes with 0, 5, 10, 50, 100 and 500 ng gentamicin per tube. Conjugate solution (50 µl diluted 1:200) is then added to each tube, followed immediately by glucose/OPD substrate solution. All of the tubes are placed on a continuous mixer for 40 minutes

- 30 -

in the dark at room temperature. After 40 minutes incubation, 125  $\mu$ l aliquots are withdrawn from each tube, placed in the wells of a microtitre tray and their optical densities measured at 405 nm with a standard plate reader (Uniskan (Trade Mark)).

A calibration curve derived from this assay showed variation of colour development from substantially maximum to substantially minimum within the range 2-500 nanogram/tube gentamicin, with an inverse relation between gentamicin level and colour intensity.

A further useful example of an assay, based on a photochemical hydrogen-peroxide-generating system, is an antiglobulin test for mouse antibody raised against E.coli 0149 lipopolysaccharide. Suitable liposomes incorporating the antigen at their surface are as described in EP 0 014 530, except that the material occluded in the liposomes is a mixture of 0.01 M FMN and 0.01 M EDTA. A sandwich-type antiglobulin test can be arranged using a commercially-obtainable conjugate of horseradish peroxidase with sheep-anti-mouse immunoglobulin, by illuminating a reaction liquid sample containing all of the ingredients including the liposomes, antibody sample, antibody conjugate, catalase, and chromogenic peroxidase substrate.

If desired, the reaction liquids can contain a suitable dense polymeric or polysaccharide suspending agent (e.g. Ficoll, (Trade Mark)) which can be provided in a suitably-chosen density of solution to eliminate the need to shake the bead suspensions.

Alternatively, the beads or other forms of polymer with the properties indicated therein can be provided in a form in which they are attached to or form part of the surface



of a macro-solid phase carrier material such as a larger (e.g. 5mm diameter) bead, or a stick, peg or strip, e.g., a paper or cellulose or glass-fibre or synthetic plastics material.

5

As alternative materials to the liposomes mentioned above, polymer membranes, especially e.g. as capsules, can be used, such as for example those described in GB 1 540 461 (Damon), and modified by attachment of specific binding groups.

10

In the examples given above, a peroxidase enzyme is used as the enzyme label attached to a binding reaction partner, and hydrogen peroxide is used as reactant for the enzyme label together with a chromogenic co-reactant.

15

Among such chromogenic co-reactants, besides 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ADBS), producing a green soluble product that absorbs light particularly strongly at 415 nm, other chromogens can be used, for example o-dianisidine, giving a sparingly soluble yellow-orange product that absorbs especially at 400 nm, 5-aminosalicylic acid, giving a brown soluble product that absorbs especially at 450 nm, dicarboxidine dihydrochloride, which gives a brown soluble product absorbing especially at 440 nm, and o-phenylene diamine, giving an orange soluble product absorbing light particularly strongly at 492 nm as well as tetramethylbenzidine, which gives a blue insoluble product.

20  
25  
30

Further examples of suitable chromogenic co-reactants are 3-methyl-2-benzothiazolinone hydrazone/3-dimethylamino benzoic acid, giving a purple soluble product absorbing especially at 590 nm, diaminobenzidine, giving a brown insoluble product, and 4-chloro-1-naphthol, giving a blue insoluble product.

35

Coloured insoluble products can be especially usefully applied to qualitative detection tests, for example.

A fluorescent result can be achieved for example by using  
5 3-(4-hydroxyphenyl) propionic acid as the co-reactant.

Alternatively, a luminescent coreactant can be employed. For example, in an embodiment of the test system described herein wherein a peroxide is present, luminol can be used  
10 to provide a detectable luminescence by reaction with the peroxide and peroxidase enzyme label.

Among the luminescent materials that can be used as a label without requiring an enzyme label are N-methylated  
15 9-acridine carboxyl groups introduced for example by reaction of the substance to be labelled with the corresponding acid halide, followed by N-methylation.

It can be seen that the features described above can be  
20 used in any desired combinations and subcombinations and in modifications or variations thereof within the scope of the invention.

CLAIMS

1. A process for carrying out a label-linked specific binding assay, e.g. an immunoassay, comprising the steps of
- 5
- providing in an aqueous reaction zone a member of a specific binding pair to participate in the assay, linked to a label which either is a first coreagent which is reactive with a second coreagent in a  
10 reaction to produce a detectable assay result, or is a generator of said coreagent,
  - providing a dispersed quantity of said second coreagent in said reaction, or of a generator of said  
15 coreagent, within a discretely bounded zone adjacent to said aqueous reaction zone and formed by a zone-forming carrier material, which also carries in immobilised form a member of said specific binding pair,
  - providing in said aqueous reaction zone a consumer of one of said coreagents in said reaction, thereby to ensure the substantial absence of said coreagent in  
20 said aqueous reaction zone,
  - wherein said discretely bounded zone comprises a barrier sufficient to prevent entry of the consumer reagent from said aqueous reaction zone into the bounded zone, thereby to separate the consumer  
25 reagent from the dispersed quantity of second coreagent or generator thereof in the bounded zone,
  - 30
  - and
  - 35 - allowing the specific binding reactions to take place whereby the assay reaction results in the binding of

- 34 -

- a variable quantity, dependent on the quantity of analyte present in the assay system, if any, of said labelled member of said specific binding pair directly or indirectly to said immobilised specific binding material, so that said detectable assay result is produced by reaction of bound label or said coreagent generated by said bound label with said coreagent in said bounded zone.
- 10 2. A process according to claim 1, wherein the discretely-bounded zone contains an enzymic or nonenzymic generator of the second coreagent.
- 15 3. A process according to claim 2, wherein the label is an enzyme and a second coreagent is a substrate for the enzyme.
- 20 4. A process according to claim 3, wherein there is also provided in the assay system a further substrate for the enzyme label.
- 25 5. A process according to claim 4, wherein the enzyme is a peroxidase, the second coreagent is hydrogen peroxide and the further substrate is a chromogenic, fluorogenic or luminescent substrate for the peroxidase, e.g. 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid), tetramethylbenzidine, 3-(4-hydroxyphenyl) propionic acid or luminol.
- 30 6. A process according to claim 1, wherein the label is a substance which can undergo a luminescent reaction and the second coreagent is a coreactant of the label in that reaction.

7. A process according to claim 6, wherein the label is an N-methylated acridine 9-carboxyl group and the second coreagent is hydrogen peroxide.

5 8. A process according to any of claims 1 to 7, in which the second coreagent is diffusible, is supplied or generated in the first zone, and its effective absence from the second zone is assured by a consumer reagent in the second zone that effectively consumes the second  
10 coreagent diffused from the first zone, thereby to prevent it from undergoing appreciable or substantial reaction with the label in the second zone.

15 9. A process according to claim 8, in which there is used a biphasic or two-compartment system in which the diffusible co-reagent is supplied or provided by a generating system in the first phase or compartment and diffuses into the second phase or compartment where it is consumed by the consumer reagent.

20 10. A specific binding assay process according to claim 1 or 9, in which (1) a gel particle or body containing a reactant-generator system for the second coreagent (such as a mixture of glucose and glucose oxidase, to make  
25 hydrogen peroxide) has immobilised specific binding agent on its surface (e.g. immobilised antibody), and is surrounded by a reaction medium containing a consumer reagent to consume any reactant that may diffuse away from the gel (such as catalase to consume the hydrogen  
30 peroxide); in which (2) the enzyme of the labelled binding material is capable, together with other reagent(s) in the system, of forming a detectable product when it comes into reactive contact with the second coreagent (eg. the enzyme is a peroxidase and a chromogenic peroxidase substrate is  
35 provided to provide a colour result when these reagents come into effective contact with hydrogen peroxide); and

- in which (3) the specific binding event of the assay results in a (variable) extent of binding (according to the quantity of analyte in the system, if any) on the part of the enzyme-labelled binding material, to the
- 5 complementary binding agent immobilised at the surface of the gel, so that the enzyme component of any material which does bind in this way comes into effective contact with the reactant and thereby forms the said detectable product.
- 10
11. A process according to claim 1, wherein polymer gel material, e.g. in the form of beadlets, having glucose oxidase entrapped therein (in the presence of glucose and oxygen), is provided with immobilised antigen or antibody
- 15 or hapten on its surface to form the first zone, thereby to generate hydrogen peroxide in the first zone, as the second coreagent, and wherein catalase or a peroxidase is provided in the second zone to consume hydrogen peroxide which has diffused there without giving rise to a
- 20 detectable product.
12. A process according to claim 1, wherein liposomes having glucose oxidase (in the presence of glucose and oxygen) or a photochemically-activatable
- 25 hydrogen-peroxide-generating system entrapped therein, are provided with immobilised antigen or antibody or hapten on their surfaces to form the first zone, thereby to generate hydrogen peroxide in the first zone as the second coreagent and wherein catalase or a peroxidase is provided
- 30 in the second zone to consume hydrogen peroxide which has diffused there without giving rise to a detectable product.
13. A test kit for carrying out a specific binding assay,
- 35 e.g. an immunoassay, the test kit comprising

- a labelled member of a specific binding pair that participates in the binding reaction of interest in the assay, linked to a label material which either is a first coreagent which is reactive with a second coreagent in a reaction to produce a detectable assay result, or is a generator of said first coreagent,  
5
- a bounded disperse reagent preparation which comprises a zone-forming carrier material which contains within a discretely-bounded zone a dispersed quantity either of said second coreagent in said reaction, or of a generator of said second coreagent, said bounded disperse reagent preparation also carrying in immobilised form at the zone boundary formed by said zone-forming carrier material a member of said specific binding pair, and  
10  
15
- a consumer reagent capable of consuming one of said coreagents,  
20
- wherein the zone boundary formed by said zone-forming carrier material provides a barrier sufficient to prevent entry of the consumer reagent into said discretely-bounded zone, thereby to separate the consumer reagent from the dispersed quantity of second coreagent or generator thereof in said zone,  
25
- wherein the bounded disperse reagent preparation and the labelled material and the consumer reagent are arranged so that they can be put into contact during performance of the assay, with the labelled material and the consumer reagent dispersed in an aqueous zone adjacent to the bounded disperse reagent preparation, whereby in use the consumer reagent ensures the substantial absence of one of said coreagents in said aqueous reaction zone, and the assay reaction results  
30  
35

in the binding of a variable quantity of said labelled material directly or indirectly to said immobilised binding material, so that said detectable assay result is produced by reaction of said  
5 coreagent in said bounded disperse reagent preparation with said label bound thereto or with coreagent generated by said label.

14. A test kit according to claim 13, wherein said first  
10 reagent preparation comprises a preparation of beads or capsules to provide the first zone, carrying immobilised specific binding material and containing a hydrogen peroxide generating system as coreagent-generating system, and wherein said second reagent preparation comprises  
15 specific binding material linked to a peroxidase enzyme or a peroxide-reactive chemiluminescent label material, the test kit also comprising catalase, (thereby in use to consume any hydrogen peroxide in the second zone outside the beads or capsules), and optionally also a chromogenic  
20 or fluorogenic or luminescent material to give a detectable result upon reaction with the label and the reactant.

15. A process according to any preceding claim,  
25 substantially as described with reference to any of the several features of the foregoing specification and Examples.



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 85/00428

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> : G 01 N 33/53; G 01 N 33/58; G 01 N 33/546																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC<sup>4</sup></td> <td style="padding: 5px;">G 01 N 33/00</td> </tr> </table> <div style="border-top: 1px solid black; padding-top: 5px; margin-top: 10px;">           Documentation Searched other than Minimum Documentation            to the Extent that such Documents are Included in the Fields Searched <sup>8</sup> </div>			Classification System	Classification Symbols	IPC <sup>4</sup>	G 01 N 33/00											
Classification System	Classification Symbols																
IPC <sup>4</sup>	G 01 N 33/00																
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>9</sup></th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0075379 (SYVA CO.) 30 March 1983, see the whole document --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-6,8-11, 13-15</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">GB, A, 2019562 (SYVA CO.) 31 November 1979, see page 2, line 7 - page 4, line 51; table 1; page 8, lines 2-37; page 27, line 1 - page 34, line 53; claims 1,3-15,18-36, 46-49,56-58 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-6,8-11, 13-15</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Clinical Chemistry, volume 29, nr. 8, August 1983, Washington, (US) I. Weeks et al.: "Acridinium Esters as high-specific-activity labels in Immuno- assay", pages 1474-1479, see page 1474, lines 30-47 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">5-7</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Chemical Abstracts, volume 96, nr. 21, 24 May 1982, Columbus, Ohio, (US), page 376, abstract nr. 177453h &amp; AU, A, 517453 (BOEHRINGER MANNHEIM GMBH) 30 July 1981, see the whole abstract --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">5</td> </tr> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	EP, A, 0075379 (SYVA CO.) 30 March 1983, see the whole document --	1-6,8-11, 13-15	X	GB, A, 2019562 (SYVA CO.) 31 November 1979, see page 2, line 7 - page 4, line 51; table 1; page 8, lines 2-37; page 27, line 1 - page 34, line 53; claims 1,3-15,18-36, 46-49,56-58 --	1-6,8-11, 13-15	A	Clinical Chemistry, volume 29, nr. 8, August 1983, Washington, (US) I. Weeks et al.: "Acridinium Esters as high-specific-activity labels in Immuno- assay", pages 1474-1479, see page 1474, lines 30-47 --	5-7	A	Chemical Abstracts, volume 96, nr. 21, 24 May 1982, Columbus, Ohio, (US), page 376, abstract nr. 177453h & AU, A, 517453 (BOEHRINGER MANNHEIM GMBH) 30 July 1981, see the whole abstract --	5
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search             20th December 1985         </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report             23 JAN. 1985         </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">           International Searching Authority             EUROPEAN PATENT OFFICE         </td> <td style="border-bottom: 1px solid black; padding: 5px;">           Signature of Authorized Officer   <div style="text-align: right;">             G.L.M. Kruidenberg         </div> </td> </tr> </table>			Date of the Actual Completion of the International Search  20th December 1985	Date of Mailing of this International Search Report  23 JAN. 1985	International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer  <div style="text-align: right;">             G.L.M. Kruidenberg         </div>											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 94, no. 3, 19 January 1981, Columbus, Ohio, (US) K. Zaitzu et al.: "New fluorogenic substrates for horseradish peroxidase: rapid and sensitive assays for hydrogen peroxide and the peroxidase", see page 125, abstract no. 12014m & Anal. Biochem. 1980, vol. 109 (1), pages 109-113, see the whole document	5
A	EP, A, 0017908 (SYVA CO.) 29 October 1980, see abstract; page 5, line 11 - page 12, line 29; page 36, line 25 - page 41, line 17; claims	1-15

## ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00428 (SA 10735)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 21/01/86

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0075379	30/03/83	JP-A- 58062562	14/04/83
GB-A- 2019562	31/10/79	GB-A, B 2018986	24/10/79
		NL-A- 7902663	09/10/79
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		US-A- 4256834	17/03/81
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